NOVEL BIOCHEMICAL APPROACHES FOR INCREASED WOOL GROWTH AND FOR ESTABLISHING RESISTANCE TO EXTERNAL PARASITES

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INTRODUCTION

The ability to transfer genes between organisms in the form of isolated pieces of DNA provides a powerful tool for the modification of the phenotypes of domestic animals. The technology involved has been used extensively in laboratory mice and is now a widely-used and powerful research tool. Application to domestic animals is more difficult because of the longer generation times of these species and because the embryos themselves are more difficult to manipulate compared with those of mice. Nevertheless, there now exist sufficient data to support the view that the general principles of gene transfer apply equally to the major domestic species. With this established, it becomes important to identify the areas where this technology might be applied. Since one of its most important attributes is the ability to transfer gene sequences to domestic animals from a variety of organisms, an area which is being studied in detail at present is the possibility of providing such animals with imaginative new capabilities to improve their productivity, ease of husbandry and quality of end-product.

This paper describes the research in two applications of the technology, namely, the possibility of modifying the biochemistry of sheep to enable them to improve wool production efficiency and of providing sheep with a novel new way to resist blowfly strike. The first area involves the transfer of genetic material from bacteria, the second from plants. Neither would be possible by conventional breeding procedures.

THE MODIFICATION OF ANIMAL BIOCHEMISTRY

Mammals are unable to synthesise the amino acid cysteine from sources of inorganic sulphur because they lack a number of key enzymes of the cysteine biosynthetic pathway. For most animals the cysteine requirement can be readily met by the diet, but this is not always so for merino sheep which have a high demand for the amino acid to sustain wool growth (Reis et al. 1963; Reis, 1979). Since this species has an abundant supply of H_2S in parts of the digestive tract, it has been proposed that the supply of cysteine could be supplemented by providing sheep with two of the key enzymes involved in the conversion of serine to cysteine in bacteria (Ward et al. 1993; Bawden et al. 1995).

The proposed research consisted of isolating from bacteria the appropriate genes for cysteine biosynthesis, modifying them so that they were able to function in mammals, testing them in transgenic mice and finally transferring them to transgenic sheep. The research has been carried out in two independent research groups, each of whom have adopted slightly different approaches to the problem. Nevertheless, the common theme in both approaches can be summarised as follows: the enzymes needed for the pathway to operate are serine transacetylase and O-acetylserine sulfhydrylase, encoded in *Escherichia coli* by the *cysE* and *cysK* genes and in *Salmonella typhimurium* by the *cysE* and *cysM* or *cysK* genes. All of these genes have been isolated and used to prepare genes of the general detail shown in Figure 1 for the gene MTCEK1.
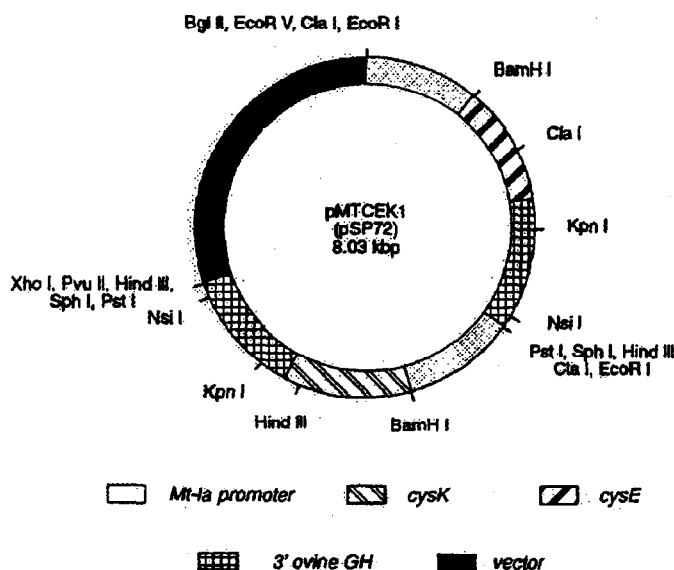
Figure 1. The structure of the plasmid pMTCEK1 which encodes the information for the biosynthesis of cysteine from serine, acetyl-CoA and H₂S. The coding sequences of the cysE and cysK genes of E. coli have each been joined to the sheep MT-Ia promoter and exon 5 of the sheep growth hormone gene.

Here, the coding sequence for each bacterial gene was independently joined to 890 bp of the promoter sequence of the sheep metallothionein-Ia (MT-Ia) gene and to portion of exon five of the sheep growth hormone (GH) gene and used to construct a single piece of DNA encoding both enzymes. Mouse L-cells transformed with the plasmid pMTCEK1 expressed the cysE and cysK sequences and cell-free homogenates from these cells could catalyse cysteine biosynthesis in vitro (Leish et al. 1993). Similar results have also been obtained using the Salmonella-derived genes under the control of different promoters (Bawden et al.1995).

In order to examine the function of the various recombinant genes described above, transgenic mice were produced by microinjection procedures. The results for the gene MTCEK1 were as follows. A total of 9 lines of mice were established, but when examined for expression of the cysE and cysK genes at the RNA and protein levels, only lines CEK-8 and CEK-28 expressed both of the bacterial genes. Line CE-8 showed the highest levels of expression and was therefore chosen for more detailed analysis.

Northern blot analysis of line CEK-8 showed appropriate RNA transcripts, which were dependent on zinc induction, a feature of transgenes under the control of the sheep MT-Ia promoter in transgenic mice. Since neither transcript could be detected in uninduced transgenic mice nor in non-transgenic mice, the gut microflora do not make a detectable contribution to the observed RNA. This is not surprising, since the cysteine biosynthetic pathway in bacteria is subject to tight transcriptional control.

Independent verification of the expression of the MTCEK1 gene in the intestinal epithelium was provided by an *in situ* analysis of the tissues of zinc-fed control and transgenic mice. The probe used for the analysis was an anti-sense RNA to the bacterial *cysE* sequence. The *cysE* mRNA could only be detected in the cells of intestinal epithelia from the transgenic mice (Ward et al; 1994). The transcripts were located in the cytoplasm of these cells, and interestingly, were usually found only in a subset of the total intestinal epithelial cell population.

Table 1. Activities of serine acetyltransferase and o-acetylserine sulfhydrylase in various tissues of transgenic mice containing the gene MTCEK1.

	Intestine	Kidney	Liver	Skin
SAT	4522	105	9	139
OASS	9642	197	11	not detected

Transgenic mice from line CEK1-8, given 25 mM $ZnSO_4$ in their drinking water for a minimum of 5 days, were sacrificed and the activities of serine transacetylase (SAT) and O-acetylserine sulfhydrylase (OASS) were assayed in intestine, kidney, liver and skin as described in (Leish et al. 1993). Values are expressed as umoles substrate utilised/mg protein/30 min. Neither enzyme can be detected in non-transgenic mice.

The activities of the enzymes SAT and OASS in various tissues of lines CE-8 and CE-28 are shown in Table 1. The highest activity was found in the small intestine with lower activities in kidney, liver, brain and skin. The high activity in intestinal tissue is in accord with previous studies on the expression in transgenic mice of a sheep growth hormone (GH) transgene under control of the MT-Ia promoter and can be ascribed in part to the oral administration of the inducer, zinc. Similar results have been reported by Palmiter et al. (1986) for the expression of GH genes controlled by the mouse MT-I promoter. We were unable to detect any SAT or OASS activities in tissues obtained from non-transgenic animals. This excludes the possibility that the enzyme activity measured in the intestinal homogenates derived from residual bacteria of the intestinal microflora, since this would be expected to be essentially identical in transgenic and non-transgenic animals.

The presence of the relevant enzymes SAT and OASS in the intestinal epithelia suggested that if such tissue could be provided with the substrates necessary for the pathway, cysteine synthesis should be possible. Since the cells already contain both serine and acetyl-CoA, it should only be necessary to provide sulphide in order to allow the biosynthetic pathway to proceed. Accordingly, a series of experiments were carried out according to the following protocol. ^{35}S-labelled Na_2S was included in a simple phosphate-buffered Krebs-Ringer incubation medium containing the dissected, rinsed intestinal tissue. After appropriate incubation, the medium in which the tissue was incubated was collected and treated with performic acid to oxidise cysteine to cysteic acid. The amino acids were separated by paper electrophoresis and the paper then cut into small strips after which radioactivity was measured in each strip. The results are shown in Figure 2.

When extracts from intestinal tissue from transgenic animals were examined, a substantial peak of radioactivity was routinely observed in the position to which cysteic acid migrates. This radioactive peak was never observed in extracts from non-transgenic mice. When eluted from the paper and analysed on an amino acid analyser, the

only amino acid that could be detected was cysteic acid and the amino acid peak was coincident with the peak of radioactivity in the sample. We interpret these results as demonstrating the *de novo* biosynthesis of cysteine from serine and H_2S by the dissected intestinal epithelium. The majority of the cysteine was found secreted in the incubation medium, a result that might be expected since the intestinal epithelium *in vivo* rapidly secretes nutrients to the portal blood supply. Radiolabelled cysteine was also readily detectable in the actual intestinal epithelium itself but not in the same quantities as those observed in the incubation medium.
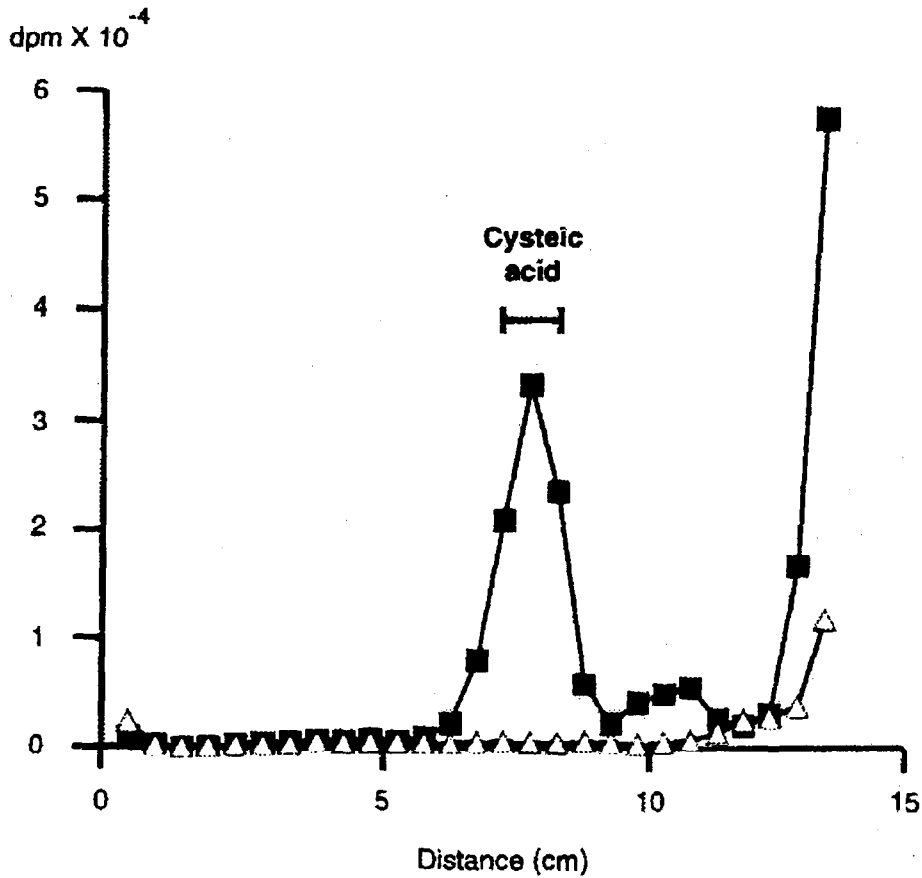

Figure 2. The biosynthesis of cysteine from H_2S <u>in vitro</u> *in intestinal tissue isolated from transgenic mice containing the gene MTCEK1. Small intestine (200 mg) from transgenic and non-transgenic mice was dissected, rinsed with 50 mM Tris buffer (pH 7.4) and incubated in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4 at 37°C for 5 min. $Na_2^{35}S$ (5 uCi) was added and incubation continued for a further 15 min. The tissue was then removed and the incubation medium treated with 100 ul of diothiothreitol (100 mM) and 3 ml of performic acid. The tissues were then homogenised and radioactive cysteine determined.*

The most critical test of the efficacy of the new biochemical pathway in producing levels of cysteine which can be used by the host would be a demonstration that a dietary cysteine deficiency could be supplemented *in vivo*. To test this, transgenic and control mice were placed on a synthetic diet in which the sulphur amino acid content was reduced to very low level (0.1% w/w of total diet) but which was supplemented with Na_2S. After 7 days on this diet, extensive loss of hair was observed on non-transgenic animals but not on the transgenic mice containing the new cysteine biosynthetic pathway. These results demonstrate that not only can cysteine be produced by the small intestine of these animals but that it can be produced in sufficient quantity to correct a dietary-induced loss of hair.

Having established that the overall concept can operate effectively in laboratory animals, the next phase of the work involves the transfer of the genes to transgenic sheep. While the actual transfer of the genes has been accomplished successfully in several experiments to date, obtaining animals in which gene are fully-functional is proving to be more of a challenge that might have been expected on the basis of the results from the transgenic mouse studies. Thus, in the case of the gene MTCEK1, three transgenic sheep have been produced that contain the pathway, but none of these has expressed the gene to allow the biosynthesis of the amino acid. Some limited expression of the genes has been obtained by others using different genes and promoter sequences and involving a total of 23 transgenic sheep (Bawden et al. 1995), but the expression levels to date have not been sufficient to alter the phenotype of the transgenic sheep. These results indicate that there may be some difference in control of the transgene in transgenic sheep compared to transgenic mice, but the numbers at present are far too low to be allow any conclusions to be made. During the current year, a larger number of transgenic lambs are being produced. Nevertheless, the conclusion is clear that when the pathway is finally optimised in sheep, substantial effects on wool growth might be anticipated under conditions where cysteine is a dietary limitation.

DISEASE RESISTANCE

A second area in which genetic engineering might be expected to play a significant role is in the prevention of animal diseases. The major diseases of domestic animals involve a significant economic cost to the farmer and it is, therefore, not surprising that disease control is a major preoccupation of all producers. A range of control measures have been adopted over the years, broadly divided into chemical, immunological and genetic techniques. They have been successful in reducing the incidence and severity of many of the highly-destructive diseases, but each technique has associated drawbacks to its universal or long-term applicability.

Chemical control methods involve both man-made chemicals and naturally-occurring compounds. Both can be associated with problems of residues in the animal and the pasture, variable rates of biodegradation and some level of toxicity to the animal itself. In addition, the target organisms often develop resistance to the chemicals, resulting in a need for increased dosage and frequency of application in order to maintain control. Chemicals are also recognised as potentially damaging to the environment, particularly if used indiscriminately, and they have frequently been implicated in widespread ecological damage.

Controlling disease by invoking the animal's immune system is an attractive approach because it uses the animal's natural defence system and provides no danger to the surrounding environment. It has proven very successful for some diseases, but may present logistic difficulties. Vaccination requires a suitable antigen, multiple inoculation of each animal to establish immunity and boosting at intervals to maintain antibody titres.

A highly desirable approach to disease control is to breed resistant animals. In this case, the animal requires no specific husbandry and the resistance is passed to succeeding generations. For example, using a conventional selective breeding approach, sheep have been selected that show increased resistance to fleece rot and blowfly strike (Raadsma, 1991) and to internal parasites (Gray, 1991). The disadvantage of this method, however, is the

slow rate of progress in the desired resistance trait and the difficulties inherent in establishing suitable selection criteria.

Genetic engineering techniques provide a novel way to establish animals genetically resistant to disease. This new technology allows small pieces of highly characterised DNA to be inserted into the genomes of domestic animals in such a way that the DNA becomes an integral part of the genetic repertoire of the recipient animal. One of its major advantages is the ability to transfer genes without regard to interspecific barriers. Thus, genes from diverse sources can be considered for their potential to increase the resistance to specific diseases. The method is potentially faster than conventional selective breeding, although, in practice, the newness of the technology means that progress is currently very slow. In principle, it is also accurate because only the gene sequence conferring the resistance trait is transferred. In addition, multiple resistance factors can be transferred by combining the genetic information from several genes.

THE APPLICATION OF GENETIC ENGINEERING TO THE CONTROL OF BLOWFLY STRIKE

One of the more important problems faced by the wool industry in Australia is the attack of sheep by the larvae of the blowfly *Lucilia cuprina*. The adult fly lays its eggs on the skin of sheep at susceptible sites that include local skin lesions, wool contaminated by faeces and urine and wool undergoing attack by bacteria and fungi that cause fleece rot. Larvae hatch from the eggs and initially feed off the local available nutrients. As they grow and develop into second and third instar larvae, however, they become more invasive, penetrate the skin of the sheep and commence feeding from the living tissue of the animal. The physical trauma caused by this invasion is severe and, together with the accumulation of toxins released by the larvae and the tissue under attack, can result in the death of the animal if left untreated.

Flystrike can be prevented effectively by the timely application of chemical insecticides, the most effective being those based on the organophosphates and cyromazine. One of the difficulties experienced by the producer, however, is determining whether such treatment is necessary in any particular year, since flystrike occurs only under specific environmental conditions that are hard to predict in advance. If possible, it is to the producer's advantage to avoid the significant labour costs involved in treating sheep.

There are obvious benefits if sheep could be developed with a genetic resistance to blowfly strike. Attempts have been made in previous years to identify such animals and introduce resistance to flocks that inhabit regions of Australia where flystrike is prevalent (Raadsma, 1991). However, flystrike resistance is multi-factorial and its selection by conventional breeding is slow and expensive. We believe that it may now be possible, using the techniques of genetic engineering, to introduce a single gene conferring resistance into the sheep genome. Our work is still in an early stage but can be summarised both in concept and in actual progress as follows:

1.	Identification of a protein with the properties necessary to establish the desired phenotype.

In order to establish resistance in sheep to blowfly strike, it was necessary to identify proteins that would inhibit the growth and development of fly larvae but at the same time exhibit no toxicity towards the sheep itself. Of the areas of fly larval morphology and biochemistry that might be uniquely sensitive to attack, the larval cuticle presents some attraction because of its high chitin content. This polymer is composed of beta-1,4-linked N-acetylglucosamine subunits and is one of the most abundant polysaccharides in nature (Flach *et al.* 1992). Chitin is not found in mammals but forms a vital part of insect cuticle, insect peritrophic membranes and fungal cell walls.

Chitin is specifically degraded by the chitinase family of enzymes. This family includes enzymes that attack chitin either as exochitinases (largely bacterial in origin) or as endochitinases (largely fungal and plant in origin)

(Flach *et al.* 1992). Recognising the link between the chitin component of insects and the degradative activity of chitinases, we examined a range of chitinase enzymes and other chitin-binding proteins for their ability to inhibit the growth of *Lucilia cuprina* larvae and discovered that several members of the endochitinase family possessed significant anti-larval activity. Because of the absence of chitin from mammals, this class of enzymes is, to the best of our knowledge, harmless to all animals. Therefore, we propose that it may be possible to induce flystrike resistance in sheep if the genetic information for an endochitinase could be inserted into the sheep genome in such a way that it could be expressed as an active enzyme in the skin and skin secretions.

2. Isolation of a Plant Chitinase cDNA and its Modification for Expression in Transgenic Sheep.

The genetic information encoding a chitinase enzyme was isolated from the plant *Nicotiana tomentosiformis* as a cDNA clone (Neale *et al.* 1990). In order to obtain adequate expression of any cDNA sequence in transgenic animals it is necessary to provide the correct promoter sequence at the 5' end of the gene and also to modify the 3' end of the sequence to provide for polyadenylation of the transcribed RNA. Since a cDNA molecule, like the coding sequences of bacterial genes, contains no introns, we employed a strategy that has proven effective in directing the expression of a range of bacterial genes. This technique uses the promoter sequence of the sheep metallothionein-Ia gene (MT-Ia) and a portion of the last exon of the sheep growth hormone gene. The structure of the recombinant gene encoding the plant chitinase is similar in overall structure to the genes previously constructed encoding the cysteine biosynthetic pathway, with the plant chitinase cDNA sequence replacing the bacterial genes (see Figure 1).

3. Transfer of the novel gene to transgenic animals.

The gene encoding the plant chitinase has now been transferred to three transgenic mice. We have yet to establish the efficiency with which this gene is transcribed in these animals, but in view of our recent success with the similar genes encoding cysteine biosynthesis, we anticipate that the genes will be utilised efficiently. It remains to be determined to what extent this novel function can confer insect resistance in such animals.

GENERAL IMPLICATIONS

Applicability

It is apparent that the expression in transgenic animals of foreign proteins with insecticidal, fungicidal or bactericidal activities offers the possibility for a novel and powerful approach to the control of disease on the farm. The primary requirement is the identification of a protein possessing appropriate therapeutic properties while invoking no adverse reaction in the host animal. The ability of genetic engineering to canvass the entire range of nature's genetic resources holds the promise of the identification of many new molecules with such characteristics. A key requirement, however, is a thorough knowledge of the structure and physiology of the target organism and the animal host in order to ensure that the selected proteins are both safe and effective. The selection of a chitinase to attack the larvae of the blowfly provides a good example. In this case, the role of chitin in the structure of the larval cuticle and the peritrophic membrane was known from fundamental studies of insect morphology and physiology. Similar basic scientific investigations had identified the mechanism of action of various chitinases on chitin substrates and the absence of any appropriate substrate in mammalian tissues.

While the attack of sheep by blowfly larvae is a serious and costly management problem, other organisms also causes difficulties to the wool grower and chitinase may also offer some protection in these areas. Lice, for example, are also a major husbandry problem on the sheep and must be removed by chemical treatment. Since lice also have a chitinous cuticle and peritrophic membrane, it is not unreasonable to expect these organisms to be vulnerable to chitinase, although this has yet to be demonstrated experimentally. Another example is that of fleece-rot, due to fungal and bacterial attack on the wool fibres themselves. Fungi possess chitin in their cell

walls and are known to be sensitive to chitinase action. It has also been demonstrated that endo-chitinases possess a low but measurable level of lysozyme activity thus providing a method for the attack of the cell wall of gram-positive bacteria. Unlike other mammals, sheep secretions are known to be deficient in lysozyme.

CONCLUSION

The concepts and results presented in this paper demonstrate the power and potential value of genetic engineering technology in the control of disease in domestic animals. Its value lies in its ability to access and use the genetic information of all species, with applications ranging from the production of specific antigens through the direct manipulation of immunoglobulin genes to the generation of unique disease resistance factors in animals. The use of foreign proteins such as plant chitinase enzymes belongs to the last category and has been discussed in detail in this paper. This example provides a particularly useful application of the technology.

REFERENCES

BAWDEN, C.S., SIVAPRASAD, A.V., VERMA, P.J., WALKER, S.K. and ROGERS, G.E. 1995. Expression of bacterial cysteine biosynthesis genes in transgenic mice and sheep: toward a new *in vivo* amino acid biosynthesis pathway and improved wool growth. Transgenic Research 4:87-104.

FLACH, J., PILET, P.-E. and JOLLES, P. 1992. What's new in chitinase research?. Experientia 48:701-716.

GRAY, G.D. Breeding for resistance to Trichostrongyle nematodes in sheep. In: *Breeding for disease resistance in farm animals*, edited by Owen, J.B. and Axford, R.F.E. Wallinford: C.A.B. International, 1991, p. 139-161.

LEISH, Z., BYRNE, C.R., HUNT, C.L. and WARD, K.A. 1993. Introduction and expression of the bacterial genes cysE and cysK in eukaryotic cells. Appl. Environ. Biol. 59:892-898.

NEALE, A.D., WAHLEITHNER, J.A., LUND, M., *ET AL.* 1990. Chitinase, beta-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. The Plant Cell 2:673-684.

PALMITER, R.D. and BRINSTER, R.L. 1986. Germline transformation of mice. Annu.Rev.Genet. 20:465-500.

RAADSMA, H.W. Genetic variation in resistance to fleece rot and flystrike in sheep. In: *Breeding for disease resistance in farm animals*, edited by Owen, J.B. and Axford, R.F.E. Wallinford: C.A.B. International, 1991, p. 263-290.

REIS, P.J. and SCHINKEL, P.G. 1963. Some effects of sulphur-containing amino acids on the growth and composition of wool. Aust.J.Biol.Sci. 16:218-230.

REIS, P.J. Effect of amino acids on the growth and properties of wool. In: *Physiological and environmental limitations to wool growth*, edited by Black, J.L. and Reis, P.J. Armidale, Australia: University of New England Publishing Unit, 1979, p. 223-242.

WARD, K.A. and NANCARROW, C.D. 1991. The genetic engineering of production traits in domestic animals. Experientia 47:913-922.

WARD, K.A., LEISH, Z., BONSING, J., NISHIMURA, N., CAM, G.R., BROWNLEE, A.G. and NANCARROW, C.D. 1994. Preventing hair loss in mice. Nature 371:563-564.